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PDGF induces osteoprotegerin expression in vascular smooth muscle cells by multiple signal pathways

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Abstract Osteoprotegerin (OPG) is a key regulator of osteoclastogenesis. Recent reports suggest that OPG may function as a protector of arterial calcification and survival of endothelial cells. However, the role and expression of OPG in vascular wall is unclear. Here we report that OPG was highly expressed in vascular smooth muscle cells (VSMC) but not in endothelial cells. Platelet-derived growth factor (PDGF), basic fibroblast growth factor, angiotensin II, tumor necrosis factor α and interleukin-1β upregulated OPG expression in VSMC. Moreover, inhibition of phosphatidylinositol 3-kinase/Akt or P38-signal pathway abrogated PDGF-induced OPG expression. Our results suggest that OPG may be an important determinant of vascular homeostasis. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Osteoprotegerin; Platelet-derived growth factor; Gene expression; Signaling pathway; Vascular smooth muscle cell

1. Intrduction

Osteoprotegerin (OPG, also known as osteoclastogenesis inhibitory factor, OCIF) is a member of the tumor necrosis factor (TNF) receptor superfamily which binds to the receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL), thereby neutralizing its functions and negatively regulate osteoclast differentiation, activity and survival [1–3]. The identification of the OPG/RANKL/RANK system as the determinant of osteoclastogenesis represents a major advance in bone biology. Targeted deletion of OPG in mice resulted in severe, early-onset osteoporosis and marked calcification of the aorta and renal arteries [3,4]. Although it has been documented that OPG is expressed abundantly in the vascular wall [5,6], the role of OPG in the vascular wall is poorly understood. In human populations, OPG is associated with a higher incidence of arterial calcification, a component of atherosclerotic lesions [7,8]. In addition, recent reports have shown that OPG administration prevents vessels from calcification induced by warfarin and vitamin D [9], and protects endothelial cells from apoptosis [10]. These findings suggest that OPG may have a protective role in the vascular system.

Platelet-derived growth factor (PDGF) is a potent mitogen and chemoattractant that functions as an important mediator in the pathogenesis of vascular disease [11,12]. Activation of PDGF receptor in vascular smooth muscle cells (VSMC) can activate several signaling pathways, including p38-, mitogen activated protein kinase (MAPK)- and phosphatidylinositol 3 (PI3)-kinase-mediated signal pathways, which transduce the signals into the nucleus and stimulate VSMC proliferation and migration [13,14].

In the present study, we document for the first time that OPG is highly expressed in human VSMC, but not vascular endothelial cells, and that PDGF induces OPG expression by multiple signal pathways including PI3-kinase/Akt- and P38-dependent pathways in VSMC. Our data implicate that OPG can function as an important determinant of vascular homeostasis in addition to bone biology.

2. Materials and methods

2.1. Materials

PDGF-BB, basic fibroblast growth factor (bFGF), angiotensin II (AngII), tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β) were purchased from Sigma (St. Louis, MO, USA). Ly294002, SB202190 and U0126 were obtained from BioMol Research Laboratories (Plymouth Meeting, PA, USA). Anti-human OPG antibody was purchased from R&D Systems (Minneapolis, MN, USA). Cell culture medium and phosphate-buffered saline (PBS) were purchased from Invitrogen (Carlsbad, CA, USA).

2.2. Cell culture and stimulation

Human aortic smooth muscle cells (HASMC) and human umbelial vein endothelial cells (HUVEC) were purchased from Biowhittaker (San Diego, CA, USA). The HASMCs were cultured in smooth muscle cell growth medium-2 containing 5% fetal bovine serum (FBS), 2 ng/ml human bFGF, 0.5 ng/ml human epidermal growth factor, 50 μg/ml gentamicin, 50 ng/ml amphotericin-B, and 5 μg/ml bovine insulin. The HUVECs were cultured in EGM-2 containing 5% FBS, 2 ng/ml human bFGF, 0.5 ng/ml human epidermal growth factor, 50 μg/ml gentamicin, 50 ng/ml amphotericin-B, and 5 μg/ml bovine insulin. For stimulation experiments, early passages (five to seven) of HASMC were grown to 80–90% confluence and made quiescent by serum starvation (0.4% FBS) for at least 24 h. The LY294002, SB202190 and U0126 inhibitors were added 30 min before the addition of human recombinant PDGF-BB, bFGF, AngII, TNFα, or IL-1β. The rat vascular smooth muscle cell line (A7r5) was purchased from ATCC (Cat# CRL-1444, Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium containing 10% FBS.

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2.3. Northern and Western blot analyses

Both Northern and Western blot analyses were performed as previously described [15].

2.4. Transient transfection and luciferase assays

Transient transfection was performed with 0.5 μg of total DNA per well and LipofectAMINE (Invitrogen) according to the manufacturer's instructions. Briefly, cultured A7r5 achieving 70–80% confluence in 12-well plates were cotransfected with 400 ng of the expression vector containing the luciferase reporter gene driven by a ~1.1 kb OPG promoter [17] and 100 ng of the green fluorescent protein (GFP) expression vector (Clontech), which was used as the control for transfection efficiency. 24 h after transfection, the cells were washed twice with PBS and subsequently cultured for 24 h in Optium-MEM (Invitrogen). After incubating with 20 ng/ml of PDGF for 6 h, the cells were assessed for luciferase activity using the luciferase assay kit (Promega). The luciferase activity was measured by a luminometer (Victor II, Perkin Elmer) and normalized by GFP level.

2.5. Adenovirus preparation and infection

Adenovirus was prepared as previously described [15]. For the generation of recombinant adenovirus encoding dominant negative IκB (named as Ad-IκBDN; Du et al., unpublished data), an IκB mutant with lysine residues at 21 and 22 were converted to arginines as previously described [18]. The virus was purified by CsCl gradient and final yields were generally 10^{10} to 10^{12} plaque-forming units (PFU)/ml. In this study, the VSMCs were infected with adenovirus vectors at ~5 PFU/cell. The cells were subjected to experiments 24–48 h after infection.

2.6. Statistical analysis

Each experimental condition was tested in triplicate, and each experiment was repeated a minimum of three times. Statistical analyses were performed by ANOVA or unpaired 2-tailed Student *t*-test. Data are presented as mean ± S.E.M. The value for $P < 0.05$ was considered significant.

3. Results

3.1. Expression of OPG/RANK/RANKL in HASMC and HUVEC

To explore whether the OPG/RANK/RANKL system exists in vascular cells, we firstly measured the mRNA levels of OPG, RANK and RANKL in HASMC and HUVEC by Northern blot analyses, respectively. As shown in Fig. 1, OPG was highly expressed in HASMC, whereas it was undetectable in HUVEC. Although RANK can be detected in HASMC and HUVEC, its mRNA levels were quite lower than OPG. In contrast, the RANKL mRNA was undetectable in both HASMC and HUVEC by Northern blot analysis.

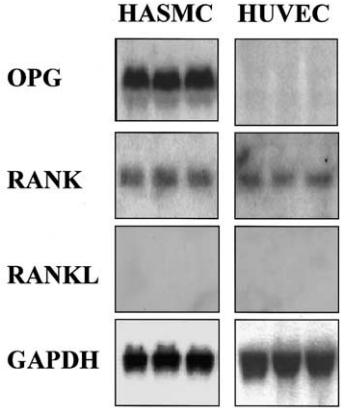

Fig. 1. The mRNA levels of OPG/RANK/RANKL in HASMC and HUVEC. Total RNA was isolated from three flasks of HASMC and three flasks of HUVEC for Northern blot analyses. Representative Northern blots show the mRNA levels of OPG, RANK or RANKL. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the control for equal loading. Three independent experiments showed similar results.

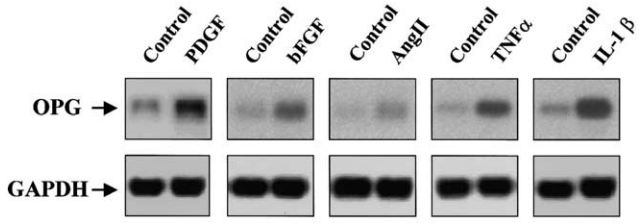

Fig. 2. OPG mRNA expression is induced by PDGF, bFGF, AngII, TNFα and IL-1β in HASMC. The cells were made quiescent by serum starvation (0.4% FBS) for 24 h, and then treated with or without 20 ng/ml PDGF, bFGF (25 ng/ml), AngII (3×10^{-7} mol/l) TNFα (10 ng/ml) and IL-1β (10 ng/ml) for 6 h. The OPG mRNA levels were analyzed by Northern blot analyses. GAPDH was used as the control for equal loading. Three independent experiments showed similar results.

3.2. Growth factors and cytokines induce OPG expression in HASMC

The growth factors and cytokines such as PDGF, bFGF, AngII, TNFα and IL-1β are critical determinants of vascular pathogenesis. To determine whether OPG expression is regulated by these factors in HASMC, quiescent cells were stimulated with PDGF, bFGF, AngII, TNFα and IL-1β for 6 h, and the mRNA levels of OPG were analyzed by Northern

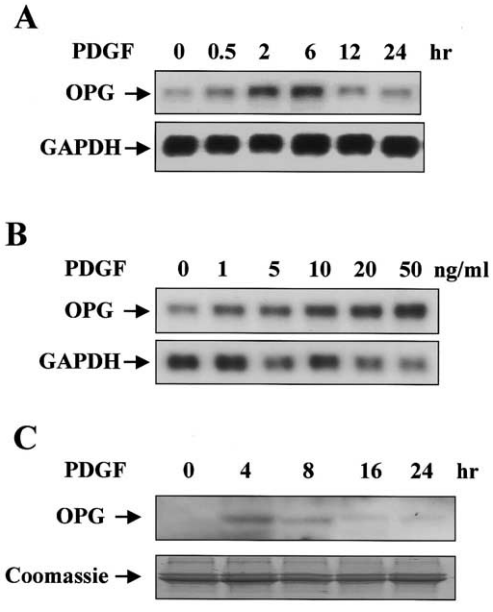

Fig. 3. PDGF induces OPG expression in a time- and dose-dependent manner in HASMC. The cells were made quiescent by serum starvation (0.4% FBS) for 24 h, and then treated with 20 ng/ml PDGF for different time periods as indicated (A) or with an increasing concentration of PDGF for 6 h (B). The OPG mRNA levels were analyzed by Northern blot analyses. C: The cell culture medium was collected and concentrated with a Biomax Column. OPG protein levels in HASMC culture medium were examined by Western blot analyses. The Coomassie blue-stained gel indicates equal protein loading. Three independent experiments showed similar results.

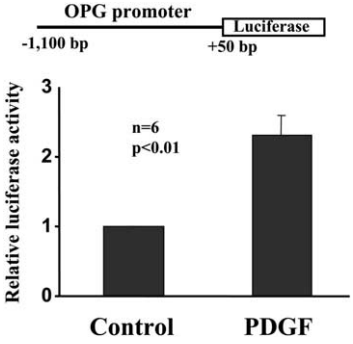

Fig. 4. PDGF stimulation activates OPG promoter. A ~1.1 kb OPG promoter/luciferase reporter construct and a GFP expression vector were cotransfected into rat vascular smooth muscle cells (A7r5). 24 h after transfection, cells were treated with Optium-MEM for 24 h and then stimulated with 20 ng/ml PDGF for 6 h. The luciferase activity normalized by GFP activity was expressed in relative units to PDGF stimulation (mean ± S.E.M., $n = 6$). An arbitrary value of 1 was assigned to GFP control.

blot analyses. As shown in Fig. 2, all of these factors used above significantly induced OPG expression in HASMC.

3.3. Time-course and dose-effect of PDGF on OPG expression in HASMC

To further confirm the effect of PDGF on the expression of OPG mRNA, different concentrations or different times of PDGF stimulation were examined in HASMC. As shown in Fig. 3A, 20 ng/ml PDGF induced the OPG mRNA expression in HASMC in a time-dependant manner which peaks at 6 h. In addition, PDGF also induced OPG mRNA expression in a dose-dependent manner (Fig. 3B). A significant increase in OPG mRNA was observed at a concentration of 10 ng/ml PDGF while the maximal increase was achieved at 50 ng/ml. Furthermore, we documented that the matured OPG protein in the culture medium of HASMC was very low and significantly increased by PDGF stimulation (Fig. 3C).

To further examine whether PDGF-induced OPG expression was regulated at transcriptional level, we transiently transfected a ~1.1 kb OPG promoter/luciferase reporter construct into rat vascular smooth muscle cells (A7r5). A GFP expression vector was used for control of transfection efficiency. As shown in Fig. 4, PDGF stimulation increased OPG promoter activity by ~three-fold.

3.4. PDGF induces OPG expression through PI3-kinase/Akt and p38/MAPK signaling pathway in HASMC

To investigate the signaling pathways mediating PDGF-induced OPG expression in HASMC, the cells were treated with 20 ng/ml PDGF for 6 h after pre-incubation with LY294002 (50 μM, a PI3-kinase inhibitor), SB202190 (25 μM, a p38 kinase inhibitor), or U0126 (10 μM, a mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK) inhibitor). As shown in Fig. 5, inhibition of PI3-kinase or p38-MAPK completely blocked the effect of PDGF ($P < 0.01$). Although inhibition of MEK significantly attenuated the effect of PDGF-induced OPG expression by 72 ± 8.5% ($P < 0.01$), U0126 alone reduced the basal level of OPG expression in VSMC by 54 ± 2.1% ($P < 0.01$). Taken together, these results suggested that OPG expression is regulated by PDGF in VSMC through several major signaling pathways including PI3-kinase/AKT and p38/MAPK.

To further confirm that the PI3-kinase/Akt pathway is involved in PDGF-induced OPG gene expression in VSMC, we selectively blocked this signaling pathway by using Ad-

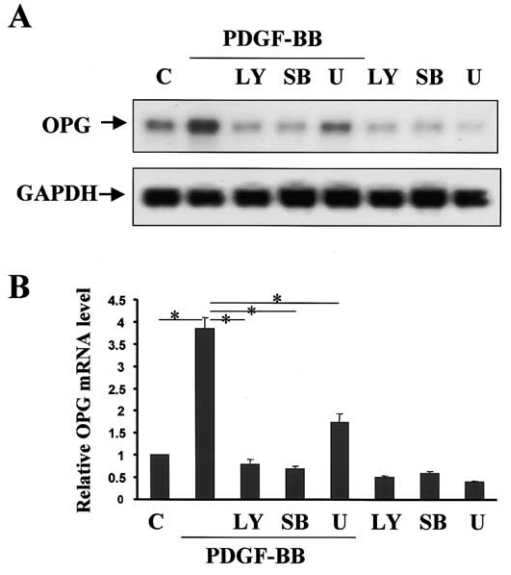

Fig. 5. Both PI3-kinase and p38 kinase inhibitor block PDGF-induced OPG expression in HASMC. The cells were treated with PDGF (20 ng/ml) for 6 h after pre-incubation with LY294002 (50 μmol/l, a PI3-kinase inhibitor), SB202190 (25 μmol/l, a p38 kinase inhibitor) or U0126 (10 μmol/l, a MEK inhibitor) for 30 min. OPG mRNA levels were determined by Northern blot analyses. A representative Northern blot (top) and the quantitative graph (bottom) of three experiments are shown. Values were normalized by GAPDH and expressed as mean ± S.E.M. ($n = 3$, $^*P < 0.01$).

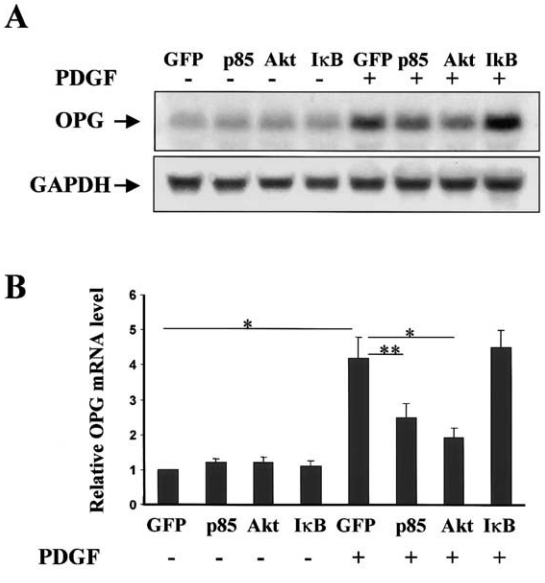

Fig. 6. Overexpression of a dominant negative p85 of PI3-kinase or protein kinase B (Akt) blocks PDGF-induced OPG expression in HASMC. The cells at 80–90% confluence were infected with the adenovirus encoding a dominant negative p85 of PI3-kinase, Akt or IκB at 5 PFU/cell for 2 h. Following 24 h in growth medium, the cells were made quiescent by serum starvation (0.4% FBS) for 24 h, then treated with 20 ng/ml PDGF for 6 h. A representative Northern blot is shown in the top panel. The average values of OPG mRNA normalized by GAPDH (bottom panel) are presented as means ± S.E.M. ($n = 3$, $^*P < 0.01$ and $^{**}P < 0.05$).

p85DN, an adenoviral vector containing a dominant negative mutant p85 of PI3-kinase as previously described [15], or Ad-AktDN, a recombinant adenovirus which contains a dominant negative form of Akt. Inhibition of the PI3-kinase/Akt pathway using this approach effectively prevented PDGF-induced OPG gene expression in VSMC (Fig. 6). However, OPG expression was not affected by the control adenovirus (Ad-GFP) infection in VSMC (data not shown). With the same approach, we used an adenovirus vector encoding a constitutively active form of IκB to examine if NF-κB is involved in PDGF-induced OPG gene expression in VSMC since NF-κB is a well-defined Akt target. As shown in Fig. 6, blockade of the NF-κB activation does not affect PDGF-induced OPG expression in VSMC. Taken together, these data documented that both PI3-kinase and Akt are involved in PDGF-induced OPG gene expression. However, NF-κB is not the downstream mediator of Akt on PDGF-induced OPG gene expression.

4. Discussion

Although OPG expression has been extensively studied in osteoblast, little is known about the regulation of OPG expression in VSMC. In the present study, we documented that OPG is highly expressed in HASMC but undetectable in HUVEC, and that RANK but not RANKL is expressed in both cells. In addition, we demonstrated that PDGF induces OPG expression in a time- and dose-dependent manner and through several signaling pathways including PI3-kinase/AKT and p38/MAPK. Furthermore, we found that multiple growth factors and cytokines such as PDGF, bFGF, AngII, TNFα and IL-1β could induce OPG gene expression in VSMC. Taken together, our data suggested that OPG plays important roles in the cardiovascular system.

The OPG/RANK/RANKL system is a critical determinant of maintenance of skeletal integrity. OPG functions as a soluble decoy receptor for RANKL and regulates bone mass by inhibiting osteoclast differentiation and activation [1–3]. However, mice with targeted ablation of OPG not only developed severe osteoporosis due to markedly increased osteoclast formation and subsequent bone resorption, but also developed profound calcification in large arteries, marked intimal and medial proliferation [3]. Thus, OPG may play a significant role in the vasculature. Indeed, a recent study showed that OPG is an important survival factor for endothelial cells [10], however, the mechanism is not clear. To date, OPG not only functions as a decoy receptor for RANKL, but also for TNF-related apoptosis-inducing ligand (TRAIL) [16]. Moreover, it has been well documented that TRAIL is secreted from VSMC and potently induces apoptosis of endothelial cells. Therefore, further studies would be interesting to examine whether OPG functions as a survival factor of endothelial cells by inhibiting the TRAIL signaling pathway.

The present study documented that OPG expression is regulated by PDGF in VSMC through several major signaling pathways including PI3-kinase/AKT and p38/MAPK. However, the downstream effectors (i.e. transcriptional factors) that directly regulate OPG transcription in VSMC are not yet identified. Using computerized analysis, we found that there are several transcriptional factor binding sites such as AP1, NF-κB and cbfa1 in the human OPG promoter (data not shown). To further characterize the downstream targets of PI3-kinase/AKT on the regulation of PDGF-induced OPG expression, we examined the role of NF-κB. However, our data suggested that NF-κB is not involved in PDGF-induced OPG expression in VSMC. We are currently investigating other transcriptional factors, but a systematic deletion mapping of PDGF-response elements in the OPG promoter may be necessary to understand this mechanism, a study which is obviously beyond the objectives of this paper.

We have documented that the OPG is expressed and regulated by growth factors and cytokines in VSMC, however, the cells used in this present study were obtained from commercially available cultured cells. What are the levels of OPG expression in normal and diseased vascular walls? To address this question, we recently examined the expression levels of OPG in the rat aorta before and after balloon-injury using quantitative reverse transcription-polymerase chain reaction. Our data suggested that OPG expression is expressed in normal vessel walls and increased after balloon-injury (data not shown). Interestingly, a recent paper documented that OPG is expressed not only in non-diseased vessel wall but also in the early atherosclerotic lesions [8]. Taken together, OPG is expressed in VSMC in vivo and may play an important role in the development of atherosclerosis.

In conclusion, the identification of potential novel functions of OPG in VSMC will definitely spur new interest in the study of OPG function. Further studies are required to elucidate the roles of OPG in the cardiovascular system.

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